Elemental Modifications and Polycyclic Aromatic Hydrocarbon Metabolism in Human Fibroblasts

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Mineral fibers and particulates represent one of the best documented, economically important, and ubiquitously occurring categories of human carcinogens. Yet, while a wealth of information exists concerning the mechanism of action of physical, chemical, and viral carcinogens, virtually nothing is known relative to the mechanism of action of this economically important class of carcinogenic compounds known as mineral fibers and particulates. While the length and diameter of various forms of asbestos have been associated with both cellular toxicity *in vitro* and tumor occurrence *in vivo*, nothing is known about whether or not these same physical properties are responsible for the purported synergistic interaction between cigarette smoking and asbestos exposure relative to the induction of bronchogenic carcinoma. Thus, while the risk of bronchogenic carcinoma for nonsmokers exposed to asbestos appears to be only slightly greater than that for unexposed nonsmoking populations, the risk of occurrence of this same tumor in asbestos workers who also smoke is approximately 100-fold greater than in nonsmoking asbestos workers. The risk of bronchogenic carcinoma is increased approximately 8-fold in asbestos workers who smoke over non-exposed smokers. Since it is clear that cigarette smoke contains over 150 polycyclic aromatic hydrocarbons, some of which are known animal carcinogens, and since other laboratories have reported that metals associated with asbestos redirect the metabolism of these agents, it was of interest to us to investigate the effects of mineral fibers on the metabolism and biochemistry of polycyclic aromatic hydrocarbons.

Introduction

Physical-Chemical Properties

Asbestos fibers represent one of the most important occupational air pollutants inhaled by miners and other exposed industrial workers. Exposure to asbestos also occurs in other areas of our society including schools and homes. Inhalation of asbestos leads to asbestosis (*1, 2*), mesothelioma of the pleura or, more rarely, of the peritoneum (*3*), and bronchogenic carcinoma (*4*). The three most commonly studied types of asbestos are chrysotile, amosite, and crocidolite. Chrysotile is the most abundant form and consists of parallel sheets of brucite and silica tetrahedrons joined in a characteristic tubular "Swiss-roll" formation with brucite forming the outer layers (*5*). Amosite and crocidolite share a common amphibole structure based on two chains of Si_4O_{11} units separated by a band of cations. However, they differ in their chemical composition, with amosite having a high ferrous iron content and crocidolite containing small amounts of Na_2O and MgO and large portions of FeO and Fe_2O_3 (*5*). The physical characteristics of these fibers are different with the amphibole fibers being straight and chrosotile curled, permitting the former to penetrate more deeply and efficiently into the respiratory bronchioli than the latter (*6*).

Biological Effects

The most commonly used biological systems for elucidation of the *in vitro* biological effects of asbestos are erythrocytes and short-termed macrophage cultures (*7*). More recently, however, fibroblast cell

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cultures have been employed to study not only cytotoxicity, but a number of biochemical and molecular parameters as well (8). This system has provided an excellent correlation between cytotoxicity and the ability of the mineral fibers tested to induce mesotheliomas following intrapleural injection in whole animals (9). As will be reported subsequently (10), the presence of heat-inactivated fetal calf serum in this system not only reduces cytotoxicity of some fibers but also reduces the effects of mineral fibers on colony morphology, giant cell formation, and number of multinucleated cells per colony being manifested. The cytotoxic effects of mineral dusts on fibroblast cell cultures differ from those seen in macrophage cultures in that silica, a relatively weak carcinogen but relatively strong inducer of fibrosis, had very little cytotoxicity in fibroblast cell cultures but was a strong cytotoxic agent in macrophage cultures (8, 11). Additionally, mineral fibers have been reported to induce giant cell formation (8), polyploidy in Chinese hamster ovary cell cultures (12), chromosomal aberrations in Syrian hamster embryo cells (12), and gene mutation at the hypoxanthine-guanine phosphoribosyl transferase loci in Chinese hamster lung cells (13). Whether these effects are the result of the asbestos alone, organic materials associated with the asbestos, or a combination of both is undetermined. The studies cited above still leave one to question whether or not the carcinogenic action of asbestos or asbestos in combination with cigarette smoking is the result of metals associated with the fibers, organic materials associated with the fibers, length and dimension of the fibers, an effect of the mineral fibers on biochemical properties such as arylhydrocarbon hydroxylase levels, the redirection of metabolism, the facilitation of transport of carcinogenic agents across the cell membrane, the interaction of charged fibers with cellular macromolecules including DNA, or the action of asbestos in modification of protein synthesis and/or lipid synthesis. It is the purpose of this paper and those which follow in this session to address some of these issues. In this particular paper we address three central issues: the uptake of chrysotile, amosite, and crocidolite into human fibroblast cultures; elemental alterations resulting from the uptake of mineral fibers into human cell cultures; and the effects of mineral fibers on the metabolism and binding of the polycyclic aromatic hydrocarbon, benzopyrene, to cellular DNA in these same cultures.

Methods and Materials

Human foreskin fibroblast cell cultures were grown in Eagles minimum essential medium with Earles salt (EMEM) (Gibco, Grand Island, N.Y.) supplemented with $1mM$ sodium pyruvate, $2mM$ glutamine, 0.11% sodium bicarbonate, and 10% fetal calf serum (KC Biological, Inc., Kansas City, Missouri). Cells were serially passaged every 4 to 5 days at a split ratio of 1 to 2 and incubated at 37°C in a 5% CO_2 atmosphere.

Electron Microscopy

Cells to be used for electron microscopic studies were treated with varying concentrations of mineral fibers for 24 hr at 37°C at a confluency level of 100% in 75 cm^2 plastic flasks (Corning). At the completion of the incubation period, the medium was decanted and the cells were washed three times with incomplete EMEM, scraped, centrifuged at 600 g for 15 min at 4°C and the resultant cell packs suspended in 0.2M cacodylate buffer to be processed for electron microscopy. After prefixation for 2 hr in 2% gluteraldehyde and washing with cacodylate buffer containing 0.1M sucrose, these cells were postfixed with osmium tetroxide (1.3%) for 1 hr at 4°C. Dehydration was accomplished with ascending concentrations of ethanol and finally propylene oxide. The specimens were exposed to a 1:1 propylene oxide 812 resin mixture and embedded in 100% 812 subsequent to an overnight exposure to the resin at 4°C. Resuspension and centrifugation at 1500 rpm was required in each processing step including the final embedding.

Tem-Stem X-Ray Microanalysis

Ultramicrotomy of the specimen blocks was accomplished with an LKB-Ultratome II utilizing a diamond knife with section thickness ranging from 500 Å to 1200 Å to meet requirements for TEM and X-ray microanalysis (14). TEM specimens were post-stained with uranyl acetate and lead hydroxide. Those sections used for x-ray analysis were mounted onto Cu grids without prestaining. A light coat of carbon was evaporated onto the specimen in order to prevent charge build-up during the excitation process. Reference specimens were asbestos sandwiches of carbon and collodion mounted on carbon grids and prepared with precipitates from a standard solution of the mineral fiber. The TEM-STEM (x-ray) analysis was performed with an Hitachi HU11D and a modified JEM 100 C that had a STEM module and an EDAX EDIT system utilizing a Texas Instrument model 707 A computer. The x-ray analysis was performed at 40 kV with 5×10^{-5} A of specimen current and 40 or 100 sec counting time. Both the itinerate spectrum stripping and peak to background programs were used to calculate and compare concentration ratios of elements in each

asbestos (15, 16). In addition, concentration ratios of both intracellular and extracellular crystals were compared with that of the standard (17). Analysis of the extracellular silicates corroborated the concentration ratios of the standard crystals. An additional reference standard consisted of the zero dose inoculated monolayer cultures that were processed parallel to the principles in order to measure the same elements in the unexposed cells and the embedding media. Chlorine from the Epon also offers an internal measuring standard for comparing specimen thickness. Confidence levels of 90-95% can be achieved in the measurements because of the low original background noise and the significant P/B ratios.

Metabolism and Binding

For these studies, human foreskin fibroblast cells were cultured on 150 mm plastic (Corning) plates under conditions cited above. These cultures were treated with asbestos and/or hydrocarbon at 60-70% confluency. [G³H] benzo(a)pyrene (³H-BP and [G³H] 7,12-dimethylbenz(a)-anthracene (³H-DMBA) were purchased from the Amersham Corporation. Both were diluted to the desired specific activity (³H-BP, 1 mCi/μmole and ³H-DMBA 20 mCi/mmole) with the appropriate unlabeled material from Eastman Kodak. These preparations were purified on the day of usage by a triple development on small TLC (silica-cyclohexane:benzene, 80:20 v-v). DMBA preparations were added to the microsomal reaction and cell medium (BP) via 50 μl of an acetone solution at requisite concentration. The experimental details of the microsomal metabolism and cell DNA binding metabolism systems have been reported in complete detail in earlier publications (18, 19).

Cytotoxicity

Cytotoxicity experiments were performed by standard procedures developed in our laboratory. Briefly, 250 and 500 human fibroblast cells were plated on 100 mm plates and allowed to settle (9 hr) in EMEM with 10% FCS. The cells were exposed to the fibers and/or hydrocarbon for the predetermined time after which the cells were washed and cultured for 3-4 weeks in the same medium to allow colony development. Colonies were fed weekly with growth medium. After this time, the cells were stained with crystal violet and colonies counted on an Artec electronic cell counter.

Results

The crystal morphology of our standard samples are illustrated in Figures 1. Figure 1A illustrates the
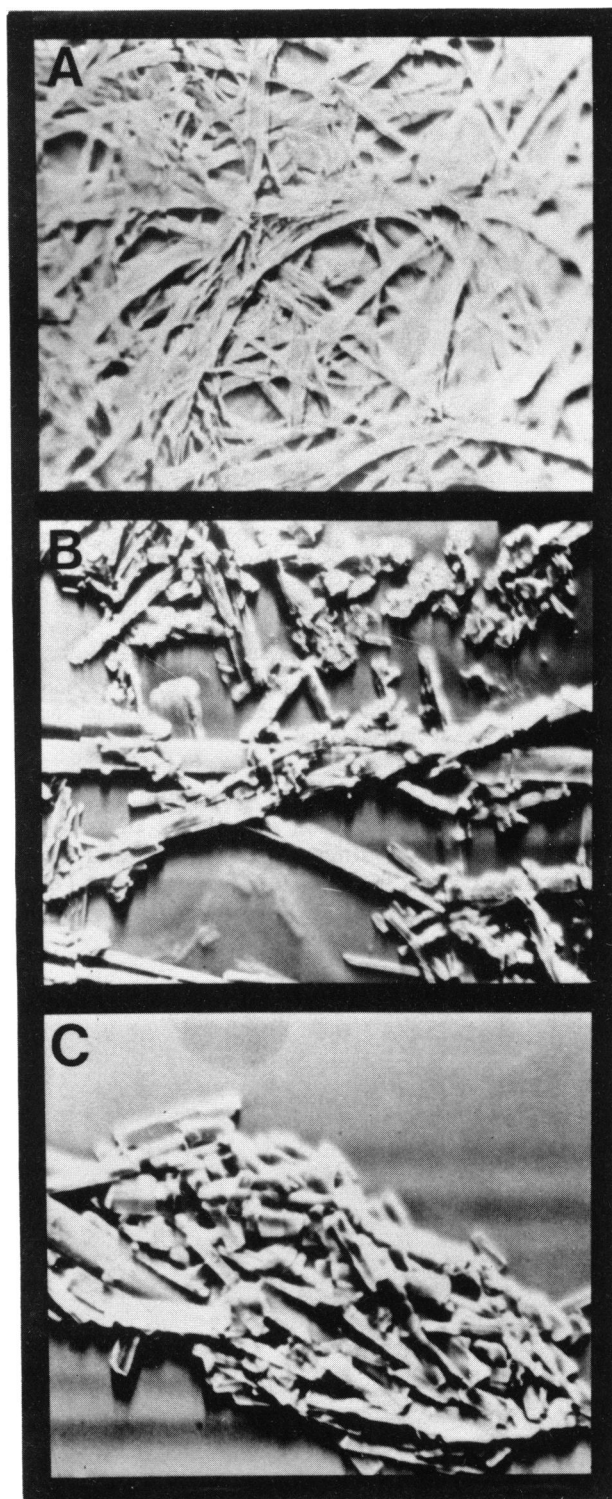

FIGURE 1. Morphology of standard samples: (A) chrysotile; (B) crocidolite; (C) amosite.
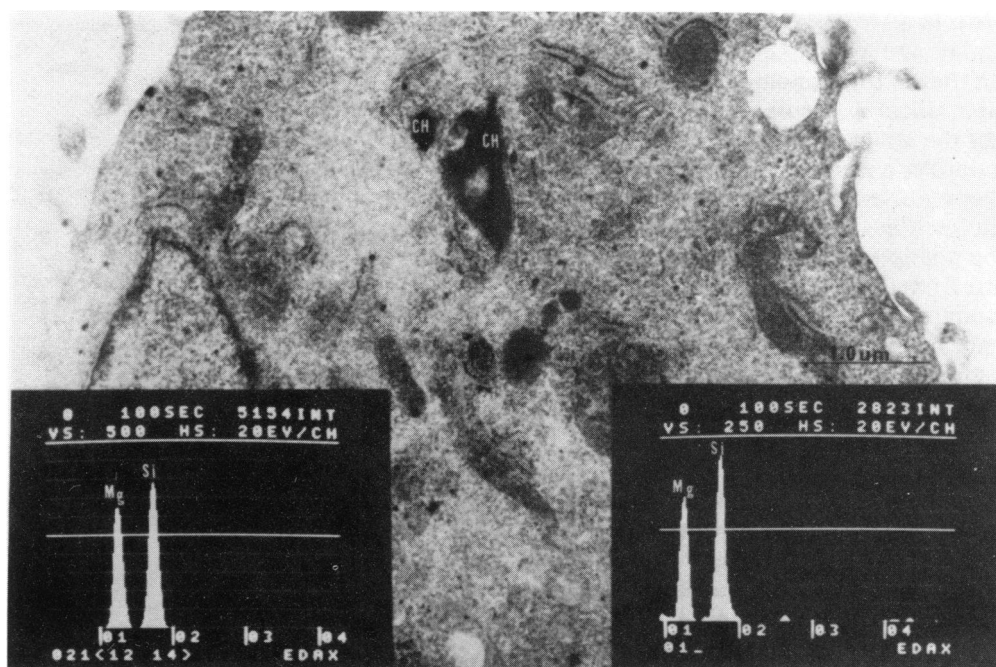
FIGURE 2. Intracytoplasmic crystal of chrysotile. Inset spectrum A represents the x-ray intensity ratios of Mg/Si in the standard sample. Inset spectrum B demonstrates the energy intensity ratios in the included crystal.

soft, silky fibrous structure of chrysotile, the most abundant form of asbestos consisting of parallel sheets of brucite and silica joined in a tubular fashion; Figures 1B and 1C illustrate the coarser and less flexible structure of crocidolite and amosite, respectively.
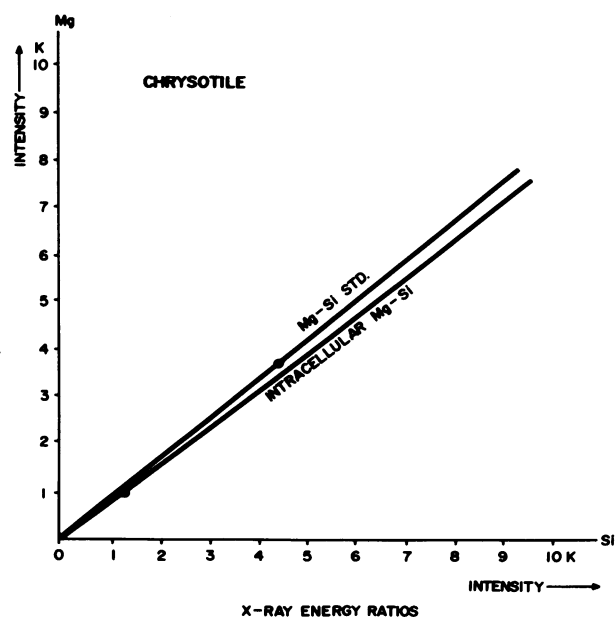

FIGURE 3. Comparative Mg/Si energy intensity ratios with those of the standard.

While no difference in x-ray energy intensity was observed between the standard and entrapped extracellular crystals, comparison of extracellular crystals with intracellular crystals however, showed that the elemental content changed upon entrapment of the fibers by the cells. Since several hundreds of cells and thousands of crystals both inside and outside of the cell were examined for each mineral fiber class studied, it appears that alteration and elemental content of the crystals reflects the working of cellular processes in a relatively short time frame of less than 24 hr. Of the three fibers examined, the NIEHS intermediate-sized chrysotile exhibited the least alteration of its elemental composition upon being engulfed. Intracellular chrysotile, demonstrated in Figure 2, contained a 1:1 ratio of Mg/Si. This is represented in the two inserts spectra of x-ray energy intensity levels. Inset spectrum A represents the standard which was shown to be equivalent to the spectrum of extracellular crystals and spectrum B the Mg/Si ratios of the engulfed crystals. The average of approximately ten random readings on standard vs. ingested material is graphically illustrated in Figure 3. Since standard deviation was less than the size of the point, error bars were not included. It is apparent from these studies that the Mg levels of the entrapped intracellular chrysotile decreased only slightly 24 hr after treatment. No other elemental change was observed. Thus, if elemental change is
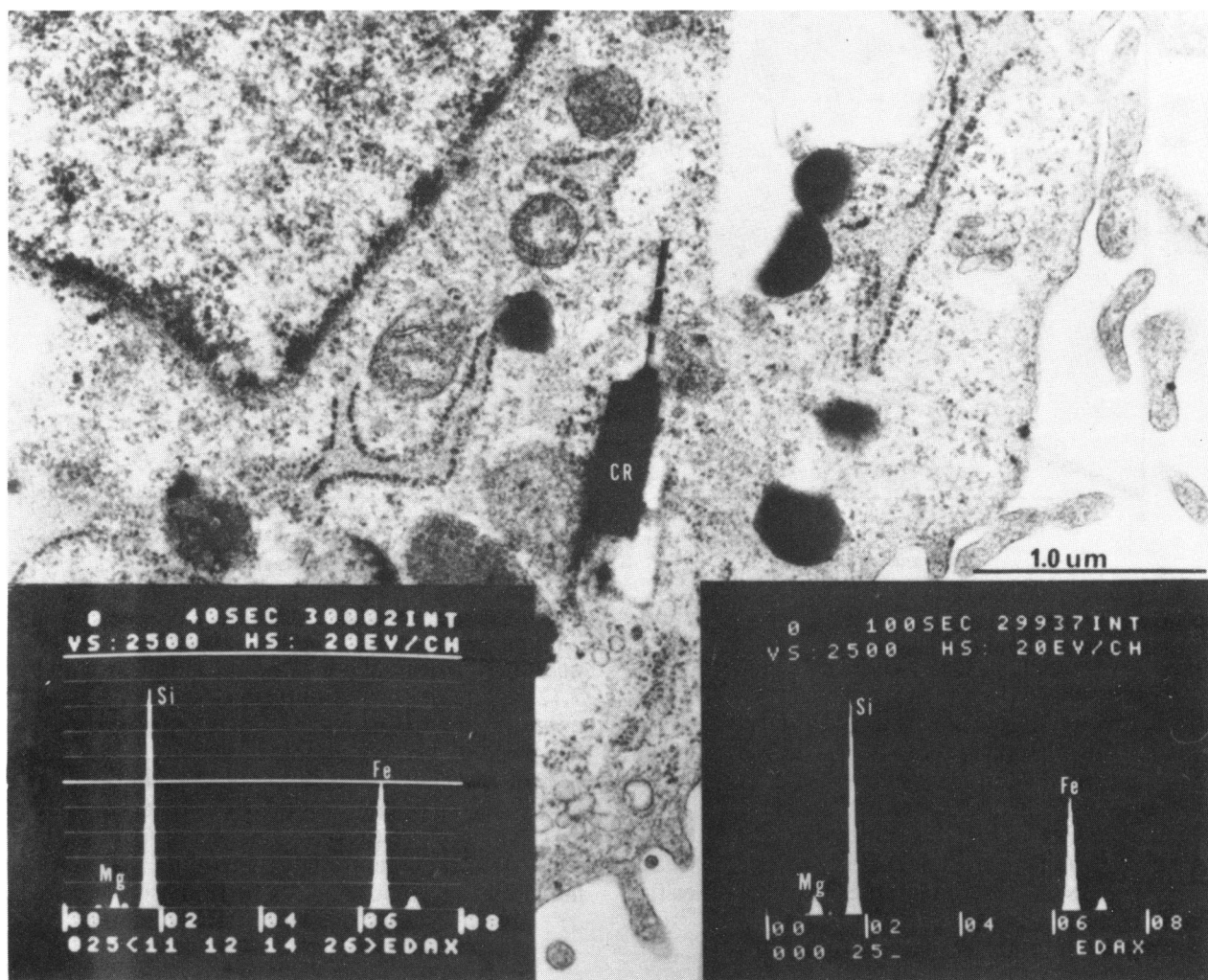
FIGURE 4. Intracytoplasmic inclusion of crocidolite: spectrum A represents the standard and spectrum B the unknown.

responsible for the observed synergism between smoking and asbestos exposure then either (1) chrysotile would not be expected to be synergistic, or if it was only slightly so, and then only at high concentrations; or (2) the time required for elemental release would exceed the time point examined. Subsequent studies however, will tend to verify the former rather than the latter explanation. As can be seen, in Figure 4 the comparative concentration ratios of Mg/Fe in silica for the standard versus ingested crocidolite reveal the slight reduction in Mg (approximately 10%) and extremely large reduction in Fe (approximately 40%). Intracellularly, within 24 hr of ingestion of the particles, inset A represents the standard control and inset B the profile of the ingested fiber; Figures 5 and 6 are the graphic demonstration of the average of approximately ten random readings each. Mg has been reported to increase the metabolic activation of polycyclic aromatic hydro-

carbons slightly, whereas Fe has been reported to be a strong stimulator of the metabolic activation of the same organic compounds (20). Amosite, like crocidolite, is a Mg-Fe silicate. However, unlike crocidolite, as can be seen by Figure 7 it often demonstrates needlelike fracture points. Intracytoplasmic inclusions of amosite measured significantly lower in the x-ray intensity ratios of Fe/Si, but unlike crocidolite the Mg/Si ratio was considerably increased in the amount of Mg. Inset A (standard curve) thereby shows a lower level of Mg than inset B (the ingested material). This observation is further emphasized in Figures 8 and 9, which represent the x-ray energy intensity differences for Mg and Fe extracellularly vs. intracellularly. These interesting results are being investigated and analyzed in even greater detail.

If indeed elemental release is responsible for the synergistic interaction of polycyclic aromatic hydro-
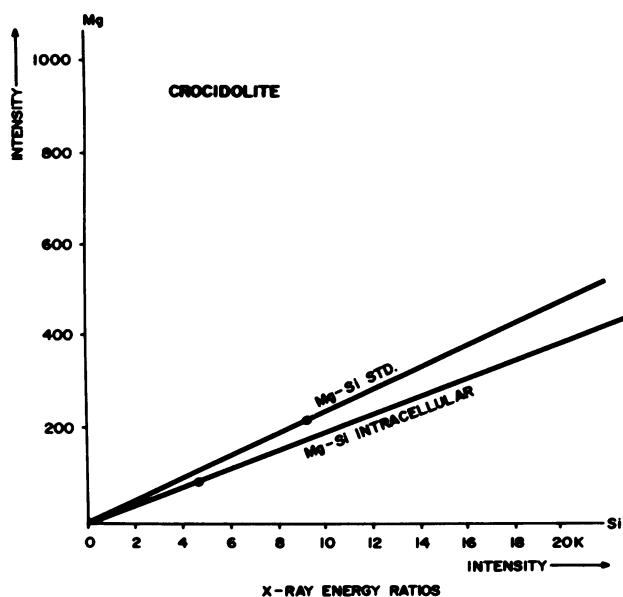
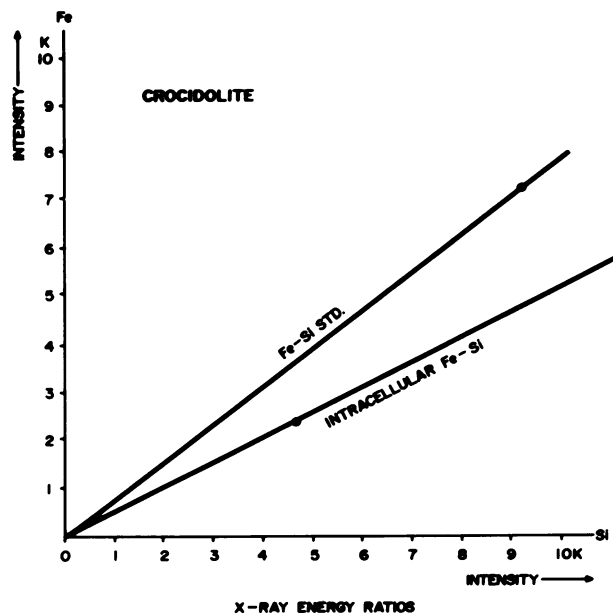FIGURE 5. X-ray intensity ratios of Mg/Si to the standard.

FIGURE 6. X-ray intensity ratios of Fe/Si to the standard.

carbons with cellular DNA via enhancement of aryl hydrocarbon hydroxylase activity, then these studies would suggest that chrysotile should not interact synergistically with the polycyclic aromatic hydrocarbons whereas amosite and crocidolite should.

The presence of NIEHS chrysotile, even at concentrations as high as 2 mg/ml in microsomal reaction mixtures, had no detectable effect on either the quantitative or qualitative nature of the oxidation of 7,12-dimethylbenz(a)anthracene. Table 1 details the extent of metabolism of ^{3}H-DMBA (as measured by the amount of water soluble radioactivity) as a function of the amount of chrysotile. Additionally, the HPLC chromatograms of the FL acetate extractable metabolites from these oxidations reveal no difference in either relative or absolute amounts of metabolites formed. Figures 10-12 show a composite
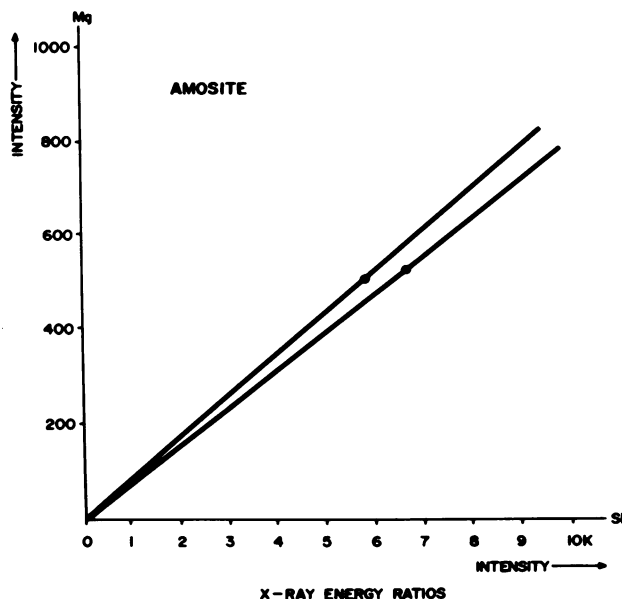
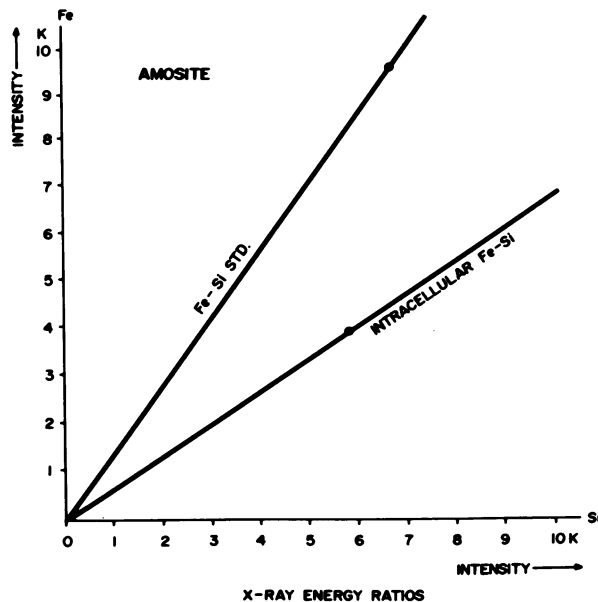FIGURE 8. Comparison of Fe/Si ratios to the standard.

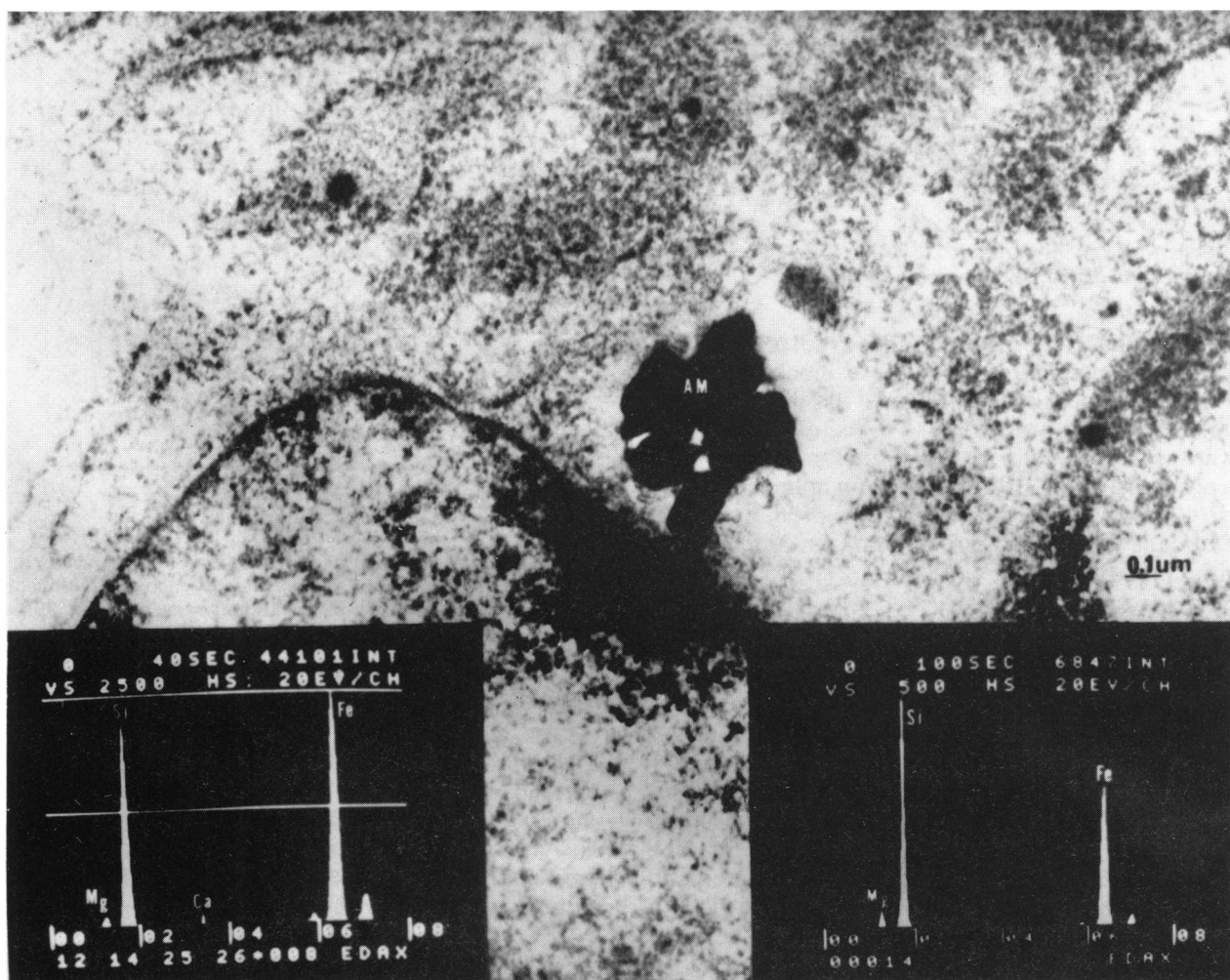FIGURE 9. Comparison of Mg/Si ratio to that of the standard.

FIGURE 7. Intracellular amosite with comparative x-ray spectra of standard (a) and unknown (b).

of HPLC traces of the ethyl acetate-extractable metabolites from three treatment groups of human foreskin fibroblast cultures, each treated with 2 μM ^{3}H-BP at either zero time, zero time concurrent with NIEHS chrysotile application at 1 μg/ml, or at zero time with 24 hr prior treatment with 1 μg/ml of chrysotile. All cultures were harvested at 72 hr for metabolite analysis and DNA isolation. As can be seen in Figure 5, the profiles are remarkably similar with respect to known metabolites, with the exception of the early eluting polar material. When the cells were treated 24 hr prior to hydrocarbon addition with 1 μg/ml of chrysotile there was a significant increase in the polar material fractions. As is demonstrated in Table 2, the amount of radiolabeled hydrocarbon bound to cellular DNA approximately was equivalent for treatment either with the hydrocarbon alone or concurrent treatment of hydrocarbon plus chrysotile. When cells were treated 24 hr prior with

chrysotile and then the hydrocarbon administered, there was a significant increase in the amount of radiolabeled bound to the DNA. These data indicate that pre-exposure of the cells to asbestos before addition of the hydrocarbon results in enhanced

Table 1. Rat liver microsomal metabolism of 7,12-dimethylbenz(a) anthracene.[a]

Chrysotile concn, mg/ml	% Metabolized to water solubility ± SD, %
0	71 ± 1.8
0.5	68 ± 2.0
1	70 ± 1.5
2.5	69 ± 2.3

[a]Reactions contained 100 nmole of ^{3}H-DMBA, 2.0 mg microsomal protein, and required cofactors (9) in a 1 ml volume buffered with 0.1 M Tris, pH 7.45, at 37°C.

Table 2. Binding of benzo(a)pyrene to human fibroblasts in vitro.[a]

| Group	Time of addition of chrysotile relative to BP	Binding level, μmoles BP/mole deoxynucleotide
A	None	0.45
B	Simultaneous	0.48
C	24 hr before	0.63

[a]Cells treated with ^{3}H-BP at 2 μM at 60-70% of confluency. Concentration of NIEHS chrysotile = 1 μg/ml.

levels of DNA binding. Our cytotoxicity experiments demonstrate that these biochemical parameters result in modification of a well established biological endpoint. Figure 13 shows that pre-exposure of the cells to chrysotile for 24 hr prior to exposure to 1 μM BP resulted in an enhancement in cytotoxicity over the levels observed by either treatment with benzopyrene alone or concurrently with chrysotile.

Conclusion

While carcinogenesis is now understood to be a multistep process, which in many cases requires metabolism, it is also apparent that the critical biochemical event in the initiation of cancer by chemicals may involve damage to the genetic material which ultimately is expressed through the action of one or more various forms of promoters. The carcinogenic potential of the polycyclic aromatic hydrocarbons has been shown to correlate well with
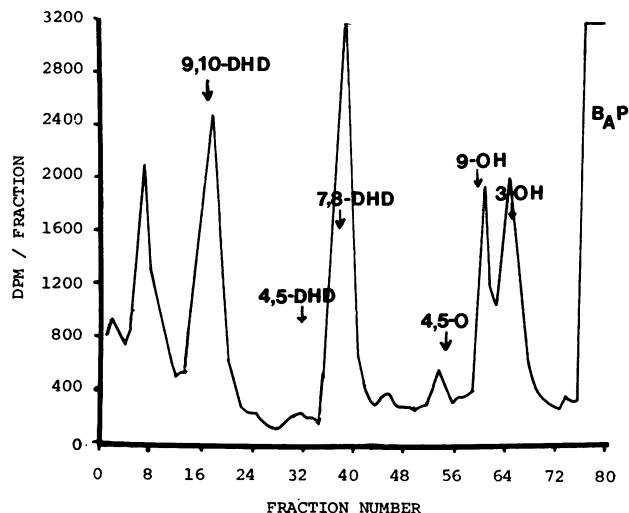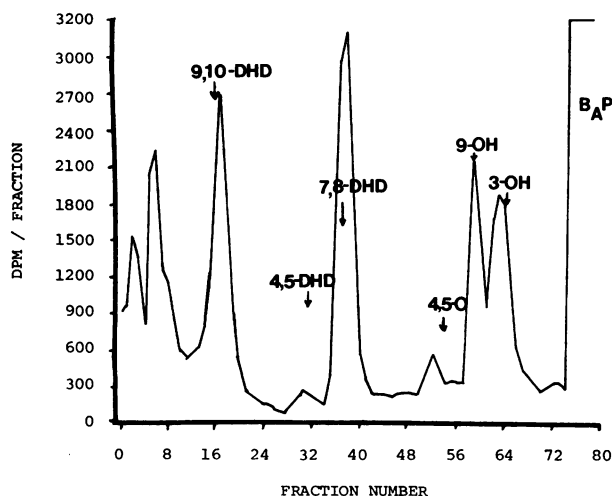
FIGURE 11. HPLC profile of ^{3}H-BP metabolites produced by human fibroblasts in vitro with simultaneous addition of 1 mg/ml NIEHS chrysotile (conditions same as in Fig. 10).

their abilities to bind to cellular DNA and when the interaction of these agents with the DNA is prevented by blocking the potential reactive site of the hydrocarbon, the agent proves to be either a weakly carcinogenic or noncarcinogenic analog of the parent compound. While it has been reported previously that AHH inducibility is increased in vivo in the lymphocytes of asbestos workers (21) and that metals associated with asbestos can modify AHH
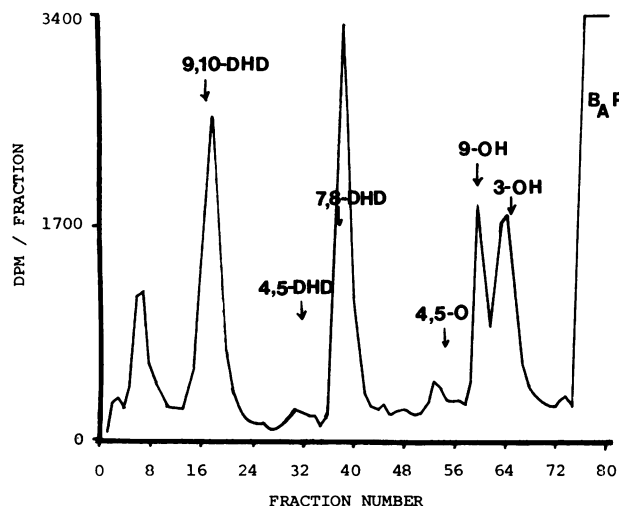
FIGURE 10. HPLC profile of ^{3}H-BP metabolites produced by human fibroblasts in vitro. Spherisorb 0.46 × 15 cm ODS column with 60% to 100% linear gradient. Fractions of 0.4 ml collected and analysed via liquid scintillation techniques.

FIGURE 12. HPLC profile of ^{3}H-BP metabolites produced by human fibroblasts in vitro when 1 mg/ml NIEHS is added 24 hr prior to ^{3}H-BP (conditions same as Fig. 10).

activity (20), our studies have shown that a form of asbestos (intermediate NIEHS chrysotile) which apparently has little elemental modification resulting from intracellular uptake enhances the binding of the potent chemical carcinogen benzo(a)pyrene to cellular DNA without the redirection or enhancement of the metabolites formed, with the exception of the more polar material. It is therefore a reasonable hypothesis, based upon these studies, that in amosite and crocidolite loss of elemental content might redirect the metabolism of the polycyclic aromatic hydrocarbons. Since there is no elemental loss in chrysotile, under the conditions used in this study, another method also must exist relating to the enhanced binding of benzopyrene to cellular DNA. A number of such possible mechanisms that could be responsible for these observations are: (1) chrysotile facilitates the transport of the hydrocarbon across the cell membrane thus increasing the amount of substrate available for enzymatic activity; (2) charged asbestos fibers interact with cellular DNA thereby perturbing the electronic structure of the macromolecule, leading to an increase in the number of sites available for binding of the BP to the DNA; (3) the chrysotile is inducing forms of metabolites not readily amenable to analysis by standard methodologies; or (4) that chrysotile redirects metabolism of benzopyrene along a different pathway, which results in metabolites of even higher DNA binding potential. While each of these possibilities exists to explain the synergistic interaction of mineral fibers with organic materials, it would be premature at this time to speculate on which of the above would be most likely. Thus, in conclusion we can only say that while there is little if any elemental alteration of chrysotile by cellular ingestion for a period of 24 hr in human foreskin fibroblast cultures, there is nevertheless a synergistic effect relative to cytotoxicity and DNA binding for chrysotile and benzopyrene in these same cell cultures when the chrysotile is added 24 hr prior to the hydrocarbon. The fact that there is such a clear relationship between the extent of DNA binding and the carcinogenic potential for polycyclic aromatic hydrocarbons is of extreme importance but may not relate, at least in the case of chrysotile, to elemental modification of the fiber itself.

FIGURE 13. Dose-survival relationship of human fibroblast cells treated with NIEHS chrysotile and/or asbestos: (O) BaP, $2mM$; (▲) NIEHS chrysotile; (●) NIEHS chrysotile plus BaP, simultaneous; (■) NIEHS chrysotile plus BaP, 24 hr prior.

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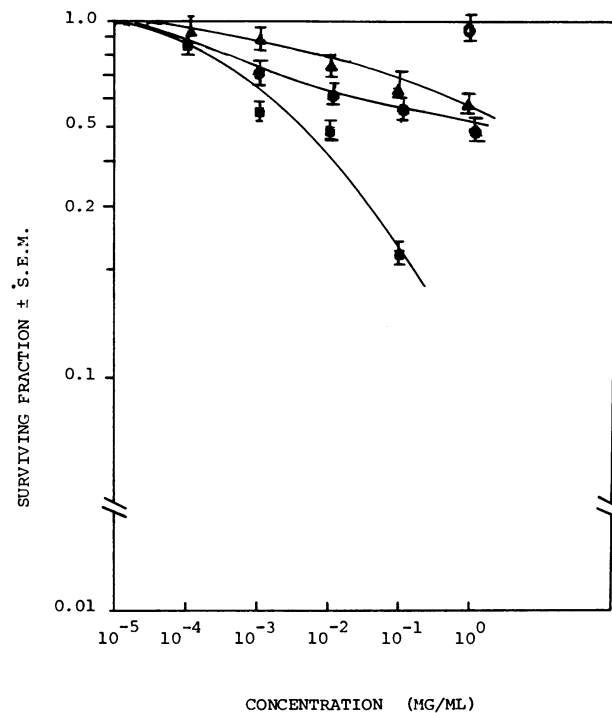2. Doll, R. Mortality from lung cancer in asbestos workers. Brit. J. Ind. Med. 12: 81 (1955).
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